

Sequence and transcript analysis of the orf140-nad3-rps12-atp1 gene cluster in the mitochondrial DNA of mung bean (*Vigna radiata* L. (Wilzek) cv. Tainan No. 5)

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Abstract. An orf140-nad3-rps12-atp1 gene cluster, which spans a 7.1 kb region of the mung bean mitochondrial genome, was identified and completely sequenced. The coding regions of nad3, rps12, and atp1 genes are highly conserved between mung bean and other plant species. The nucleotide sequences of orf140 and its 5'-flanking region are homologous with ORFBs in sunflower and *Oenothera*, and are also found to be conserved in several chimeric ORFs of various plant CMS lines. The region 3'-downstream from the mung bean atp1 is similar to its counterparts in soybean and common bean, which has been shown to be specific to a subset of legumes. Genes of this cluster are present as a single copy in mung bean. Northern-blot hybridization and RT-PCR suggested that the four genes are co-transcribed and then processed into transcripts of different sizes. Variation of accumulation levels of different transcripts was observed.

Keywords: DNA sequence; Mitochondrial gene cluster; Mung bean; orf140-nad3-rps12-atp1; Transcription.

Introduction

The higher plant mitochondrial (mt) genomes, ranging in size from 208 to 2,400 kb, are much larger and more complicated than their counterparts in animals and fungi (Palmer and Herbon, 1987; Ward et al., 1981). In spite of variation in genome sizes, most plant mt genomes encode roughly the same complement of gene products, including 3 rRNAs, 15-20 tRNAs, and approximately 20 polypeptides (Wolstenholme and Fauron, 1995). Although coding regions of individual genes are highly conserved, the mt genomes of higher plants exhibit distinctive gene orders in virtually every species so far examined (Palmer, 1992). The rearrangements are mainly due to recombination events between repeats of various sizes, which are dispersed throughout the plant mitochondrial genome (Small et al., 1989). Besides the effect on genome organization, frequent genomic recombinations can also create chimeric or truncated open reading frames (ORFs), affect transcriptional patterns, and place genes that are far apart in one genome into close proximity in another species (Hanson and Folkerts, 1992).

Most plant mitochondrial genes are monocistronic and separated by long spacers (Hanson and Folkerts, 1992). When transcripts are analyzed, the mRNAs are found to be generally larger than the actual coding regions and to contain extended non-coding 5'- and 3'-transcribed regions (Binder et al., 1996). Thus, genes located closely together

are inevitably co-transcribed. Examples of mitochondrial gene co-transcription have been reported in a number of plants, including maize (Gualberto et al., 1991), wheat (Gualberto et al., 1988), rice (Nakazono et al., 1995), *Arabidopsis* (Brandt et al., 1992), petunia (Pruitt and Hanson, 1989) and tobacco (Bland et al., 1986). Most arrays of co-transcribed gene clusters are specific to the mitochondria of a given plant species, except the three gene clusters *rrn18-rrn5*, *nad3-rps12*, and *rps3-rpl12*, which have been highly conserved (Palmer, 1992). The co-transcription of genes that encode proteins functioning in different pathways indicates that post-transcriptional and post-translational regulation are involved in controlling the abundance of the gene products. Furthermore, the high variability of genome organizations between different species and the complexity of possible transcription patterns make extensive sequencing and transcription mapping necessary to understand the mechanisms involved in regulation of plant mitochondrial gene expression.

In this work, we have completely sequenced a 7.1 kb region of mung bean mtDNA, which contains an unidentified ORF (orf140), nad3, rps12 and atp1 genes. We have also shown that these four genes are co-transcribed.

Materials and Methods

Plant Materials

Mung bean (*Vigna radiata* L. (Wilzek) cv. Tainan No. 5) was used as the source for mtDNA and mtRNA purification. Etiolated seedlings were grown in darkness at 30°C on vermiculite soaked in water.

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Mitochondrial Nucleic Acid Extraction and Analysis

Mitochondria were isolated from 4-day-old etiolated mung bean hypocotyls by sucrose gradient purification (Dai et al., 1991). The mtDNA was subsequently purified from DNase-treated mitochondria on CsCl-DAPI density gradient (Chiang, 1968). Total mtRNA was extracted from mitochondria as described by Stern and Newton, 1986. Southern- and Northern-blot hybridizations were all performed according to Sambrook et al., 1989.

Probes and Labeling Procedures

Probes 1-5 (Figure 1) correspond to the BamHI-SpeI, SpeI-SpeI, SpeI-EcoRI fragments from the 5.1 kb BamHI clone, and EcoRI-BamHI, SmaI-EcoRI fragments from the 2.8 kb EcoRI clone, respectively. The PCR probe, which was initially used for screening the *atp1* gene from the library, was obtained by amplifying mung bean mtDNA at the following primers: 5'-TGGAATTCTCTGTAAGAGCTG-3' and 5'-AAGCGGTAGATAGCCTGGTTC-3', which correspond to nucleotides 2-22 and 1077-1097, respectively, of the coding region of the pea *atp1* gene (Morikami and Nakamura, 1987). PCR amplification was performed commonly with 250 ng of mtDNA, 5 units of Taq DNA polymerase (Promega), 0.25 μ M dNTP in 50 μ l of the recommended buffer. The denaturation, annealing, and extension times were 1 min, 2 min, and 3 min at 94°C, 55°C, and 72°C, respectively. The cycle was repeated 30 times. DNA fragments were extracted from agarose gels (GeneClean III kit, BIO 101) and labeled by the random priming method (Prime-a-Gene kit, Promega).

DNA Cloning

Mung bean mtDNA was digested with EcoRI and cloned in pBluescript II SK(+) (Stratagene). The 2.8 kb EcoRI clone was identified with the PCR probe described above. As to the 5.1 kb BamHI clone, mtDNA was digested with BamHI, Southern-blotted, and hybridized with probe 4. The band corresponding to the hybridized 5.1 kb BamHI fragment was recovered from the gel and cloned. Positive clones were selected by screening with the same probe 4. Colony hybridization and plasmid isolation were performed according to Sambrook et al., 1989.

DNA Sequencing

All nucleotide sequences were obtained with double-stranded plasmids. Primers were synthetic oligonucleotides complementary to both DNA strands and spaced every 300-400 bases. Sequencing was performed as described by the manufacturer on the A.L.F.TM DNA sequencer (Pharmacia). Sequences were analyzed via WisconsinTMGCG.

Reverse Transcription

PCR (RT-PCR)- For RT-PCR, the following oligonucleotides were employed (for their positions, please see Figure 1): primer 1: 5'-GTGGTTAGTTCGCGAGCTCTT-3'; primer 2: 5'-AACGGCTAGCCAAGCAAGGTT-3'; primer 3: 5'-GAAAAACGGCGCACGGACCGT-3'. The first strand cDNA was synthesized from 2 μ g DNase I-treated mtRNA with primer 1 by using the Murine-MLV reverse transcriptase (Clontech). Products of reverse transcription were purified by phenol/chloroform extraction, and sub-

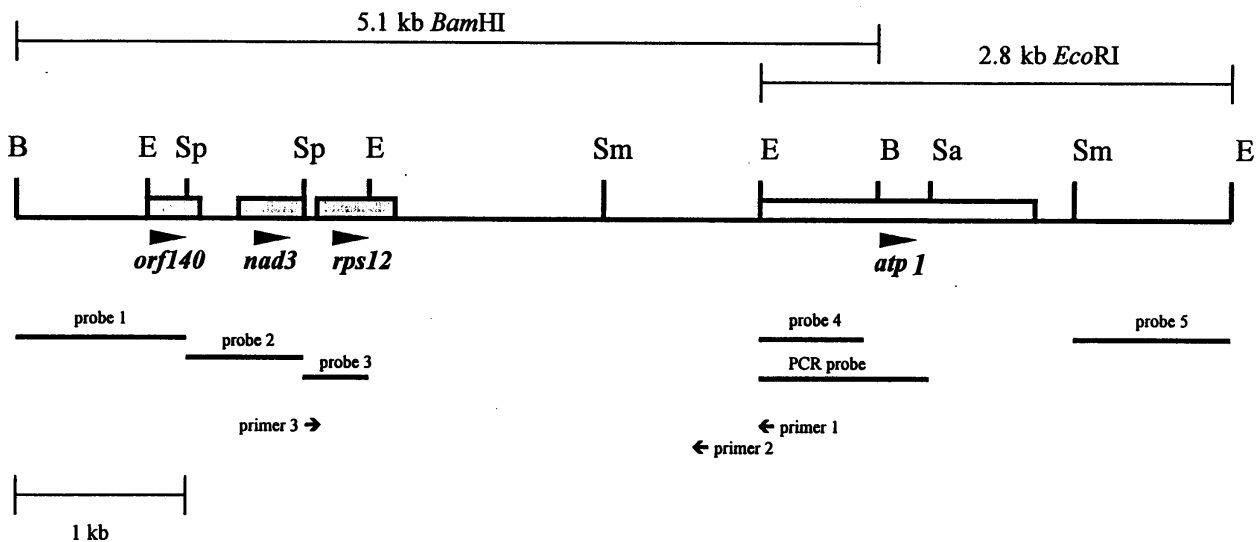


Figure 1. Genomic organization of the *orf140-nad3-rps12-atp1* gene cluster in mung bean mtDNA. Restriction recognition sites are indicated for BamHI (B), EcoRI (E), SacI (Sa), SmaI (Sm), and SpeI (Sp). The boxes indicate the coding regions of *orf140*, *nad3*, *rps12* and *atp1* genes. Arrowheads indicate their transcriptional orientations. Probes for hybridization are represented by lines below the genetic map. Arrows indicate relative positions and directions of the primers 1, 2 and 3 used for RT-PCR. The positions of cloned 2.8 kb EcoRI and 5.1 kb BamHI are indicated by lines above the genetic map.

[illegible]

Figure 2. DNA sequence comparison between the 3'-flanking regions of mt *atp1* from mung bean, soybean, and pea. Only nucleotides divergent from the mung bean are given in the latter two sequences, and identical nucleotides are indicated by asterisks. Gaps (indicated by dots) have been introduced in order to get the best alignment. The end of the coding region of *atp1* is marked by (▼). Sites of initiation codon and stop codon of *orf214* of soybean and *orf209* of common bean are indicated by (↓).

jected to two rounds of PCR amplification. PCR was performed as described above. Primer 1 and primer 3 were used in the first round of PCR. To increase specificity, 1 μ l of 100 times diluted products of the first round PCR was then used in the second round of PCR with primer 2 and primer 3.

Results

Cloning and Sequence Analysis of the orf140-nad3-rps12-atp1 Gene Cluster in Mung Bean Mt Genome

A PCR generated DNA fragment (see Materials and Methods) was used to identify the *atp1* gene from a library of EcoRI-digested mtDNA. A clone, containing a 2.7 kb EcoRI fragment, was identified and completely sequenced. The sequence showed that the 2.7 kb EcoRI mtDNA fragment contained most of an *atp1* gene and its 3'-downstream sequence. To obtain the 5'-upstream sequence region of the *atp1* gene, mtDNA was digested with BamHI and Southern-hybridized with the probe 4 (Figure 1). One signal of 5.1 kb was obtained, subsequently recovered from the gel, and cloned. Positive clones were selected by screening with the same probe 4. A clone, which contained the 5.1 kb BamHI mtDNA fragment and overlapped the EcoRI-BamHI region with the 2.7 kb EcoRI fragment, was identified and completely sequenced. In combination, the two clones cover a 7.1 kb fragment of the mung bean mtDNA (Figure 1). The sequence of this region has been deposited in the GenBank database under the accession number AF071550.

In a search for potential open reading frames (ORFs), at a discriminator setting of 100 codons, four potential genes were obtained (Figure 1). By sequence homology

to known genes, three of them were identified as nad3, rps12 and atp1 genes. As in other higher plants, the nad3 and rps12 genes of mung bean were found to be closely linked together. Similarity scores above 95%, using the FASTA program, were obtained when comparing the coding regions of these two mung bean genes with their counterparts in sunflower (Perrotta et al., 1996), petunia (Young and Hanson, 1987), and ginseng (Kim et al., 1991). The similarity was extended further into the region upstream from the nad3-rps12 ORFs for 50 nucleotides from the first methionine codon of the nad3.

The mung bean *atp1* gene was identified 2.3 kb downstream from the *rps12* gene. Analysis of the mung bean *atp1* gene showed that the coding region was most homologous with the *atp1* genes of common bean, soybean, and pea (scores 98.9%, 98.2%, and 96.8%, respectively) (Chanut et al., 1993; Chase and Ortega, 1992; Morikami and Nakamura, 1987). The nucleotide sequence of the 5'-flanking region of the *atp1* gene diverged extensively immediately outside the coding region among species. However, the 3'-flanking region of the mung bean *atp1* ORF showed extensive homology with the *orf214* and its 3' flanking region of soybean and the *orf209* of common bean, both of which are located immediately downstream from their *atp1* genes (Figure 2).

We have also identified a possible ORF (orf140) located 200 bp upstream from the *nad3* gene. Sequence alignment showed that the nucleotide sequence of the orf140 and its 5'-flanking region was highly homologous with the ORFBs located 0.5 kb upstream from the *coxIII* gene of sunflower (Quagliariello et al., 1990) and *Oenothera* (Hiesel et al., 1987) (Figure 3). However, because of the different reading frames used, the generated amino acid sequence of orf140 is completely different from ORFB, or any other identified gene.

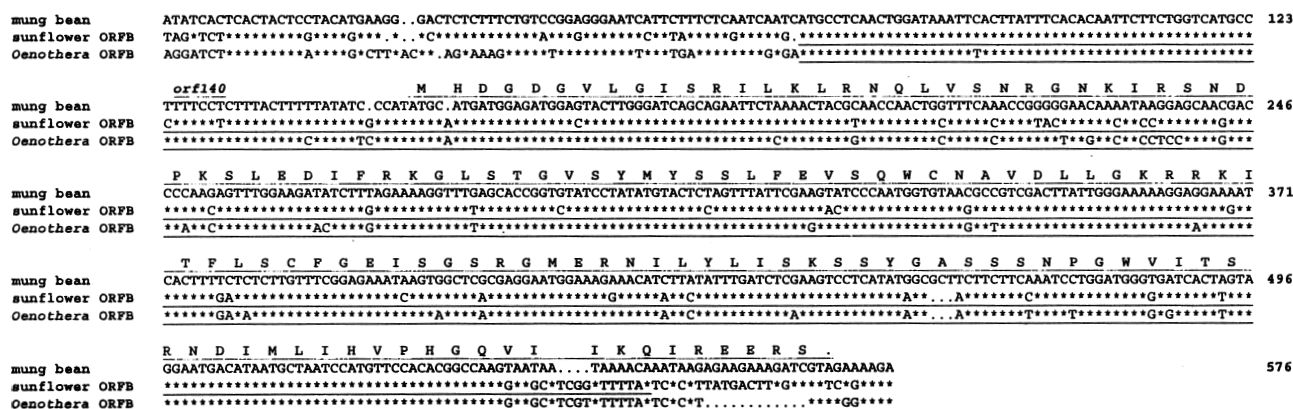


Figure 3. DNA sequence alignment of the orf140 and its 5'-flanking region of mung bean with ORFBs of sunflower and Oenothera. Only nucleotides divergent from the mung bean are given in the latter two sequences, and identical nucleotides are indicated by asterisks. Gaps (indicated by dots) have been introduced in order to get the best alignment. The putative amino acid sequence of orf140 is shown above the nucleotide sequence. The coding region of ORFBs of sunflower and Oenothera are underlined.

Besides those mentioned above, the only region within the 7.1 kb mung bean mtDNA fragment showing significant similarity with published sequences was identified in a region of the interspace between *rps12* and *atp1* (from position 2,450 to 2,700 in the deposited sequence), which was homologous with a region between *nad1* exon 3 and *orf251* of Arabidopsis mtDNA (85650-85900) (Unsold et al., 1997).

Southern-Blot Analysis of orf140, nad3, rps12, and atp1 Genes

To find out whether *orf140*, *nad3*, *rps12*, and *atp1* genes are repeated elsewhere in the mung bean mtDNA, Southern hybridization experiments were performed. MtDNA was digested with *EcoRI*, *SacI* or *BamHI* and probed with probe 4, which is *atp1*-specific, or digested with *BamHI* and probed with probes 1, 2, 3 or 5, which are specific to the 5'-noncoding region-*orf140*, *nad3*, *rps12*, and the 3'-noncoding region, respectively (Figure 1). As shown in Figure 4, single bands were detected by all of these probes. This suggests that each of the *orf140*, *nad3*, *rps12*, and *atp1* genes is present in only a single copy in the mung bean mt genome.

Transcript Analysis of the Mitochondrial orf140-nad3-rps12-atp1 Gene Cluster

To investigate transcription of *orf140*, *nad3*, *rps12*, and *atp1* genes, Northern-blot analysis was carried out on total mitochondrial RNA using probes 1-5 (Figure 1). As shown in Figure 5, transcripts ranging in size from 6.2 kb to 0.7 kb were obtained. Among these, a common 6.2 kb RNA was detected by all probes except probe 5. A 5.0 kb band was detected by probes 2-4, but not by probe 1, indicating that during the early steps of processing the first 1,200 bp of the 6.2 kb precursor is cleaved off. Correspondingly, small transcripts (1.5-0.7 kb) were de-

tected by probe 1 and probe 2, strongly suggesting that the four genes in the cluster are co-transcribed. Other detected transcripts are likely to be processed products of this 6.2 kb precursor RNA and some degradation products although the possibility that alternative sites of transcription initiation exist cannot be completely excluded and warrants investigation. A specific 2.3 kb transcript was detected by probe 2 and probe 3; however, the abundance of this RNA was much lower than the 5.0 kb transcript. Probe 4 hybridized strongly to two RNAs, 2.0 kb and 1.8 kb, both of which were much more abundant than the 5.0 kb transcript. Probe 5 failed to hybridize to any transcript significantly.

To confirm the co-transcription of *orf140*-*nad3*-*rps12*-*atp1*, DNase-treated mtRNA was amplified by RT-PCR. As shown in Figure 6, an expected 2.3 kb fragment was amplified (for primer locations, see Figure 1). No fragment was amplified when the DNase-treated mtRNA was used instead of cDNA in the PCR. The same 2.3 kb band was obtained when mtDNA was used in amplification. From the results of Northern blot and RT-PCR, we conclude that the four genes are co-transcribed.

Discussion

We have completely sequenced a 7.1 kb mtDNA fragment of mung bean, which contains an *orf140*-*nad3*-*rps12*-*atp1* gene cluster. All these genes were shown to be present as a single copy in the mung bean mitochondrial genome; thus, they must be expressed if they are functional in mung bean. In the mitochondrial genomes of *B. campestris*, maize and Arabidopsis, *atp1* is located tens of kb away from the *nad3*-*rps12* gene cluster and in different gene orientations (Makaroff and Palmer, 1987; Unseld et al., 1997). Variations in relative positions of these genes found in mitochondrial genomes of different plant species

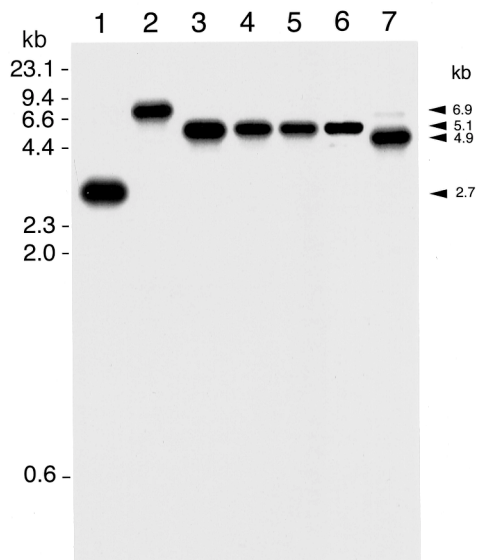


Figure 4. Southern-blot analysis of mung bean orf140-nad3-rps12-atp1. mtDNA (0.25 μ g) from 4-day-old etiolated mung bean hypocotyls was digested with EcoRI (lane 1), SacI (lane 2), and BamHI (lanes 3-7) and probed with probe 4 (lanes 1-3) and probes 1, 2, 3, and 5 (lanes 4, 5, 6 and 7, respectively). For probe locations see Figure 1. The sizes of hybridized bands are indicated on the right. Numbers on the left indicate the sizes of molecular markers (HindIII-digested λ).

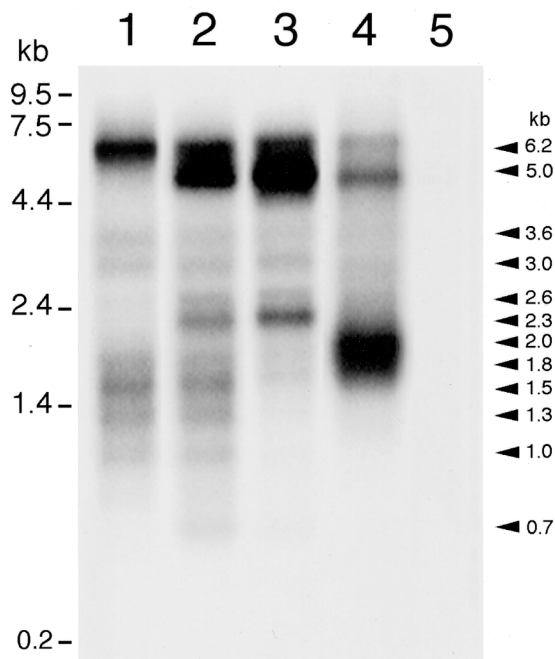


Figure 5. Northern-blot analysis of mung bean orf140-nad3-rps12-atp1. In each lane, 5 μ g of total mtRNA from 4-day-old etiolated mung bean hypocotyls was used. Northern-blot hybridization was carried out with probes 1-5 (Figure 1). Numbers of lanes correspond to those of the probes used. Sizes of RNA molecular-weight markers (BRL), ranging from 0.2 to 9.5 kb, are indicated by the numbers on the left. Numbers on the right indicate sizes of different transcripts.

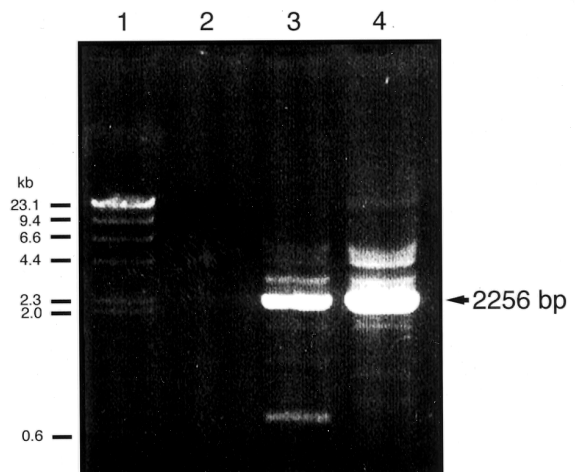


Figure 6. Amplification of the co-transcribed transcript of the orf140-nad3-rps12-atp1 gene cluster by RT-PCR from mung bean mtRNA. cDNA synthesized from DNase-treated mtRNA was PCR-amplified as described in Materials and Methods (lane 3). The PCR generated fragment was electrophoresized in a 1% agarose gel. The expected amplified 2.3 kb band from co-transcribed transcripts is indicated by the arrow. DNase-treated mtRNA (lane 2) and mtDNA (lane 4) were also PCR amplified as negative and positive control, respectively. Lane 1, size markers (DNA fragments of HindIII-digested λ).

reflect the frequent recombinatory nature of plant mitochondrial genomes. In contrast, plant mitochondrial coding sequences have been shown to be highly conserved even between evolutionarily distinct plant species (Palmer, 1992). Accordingly, the nad3, rps12, and atp1 of mung bean are highly homologous with their counterparts in other plant species. In addition, we have found that the 3'-flanking region of the mung bean atp1 gene shows extensive homology with its counterparts in soybean (orf214) and common bean (orf209) (Chanut et al., 1993; Chase and Ortega, 1992) (Figure 2). This homology seems only limited to a subset of legumes. Part of this region has also been found downstream from the 5S rRNA coding region in soybean mtDNA and probably mediates the rearrangement of the rrn5 gene in the soybean tissue-culture line SB-1 (Chanut et al., 1993). However, this region is not repeated elsewhere in the mung bean mitochondrial genome (Figure 4, lane 7) and is possibly not involved in any recombination event in mung bean.

The nucleotide sequence of orf140 and its 5'-flanking (150 nucleotides from the initiation codon) is homologous with the ORFBs of sunflower and *Oenothera* (Hiesel et al., 1987; Quagliariello et al., 1990). However, a different open reading frame is used in orf140, which could result from base duplication and/or insertions/deletions of this sequence during evolution. ORFB has been identified as homologous to atp8, its gene product essential for F_0 enzyme complex formation (Gray et al., 1998). It is possible that atp8 in the mung bean mitochondrial genome could

have shifted to the nucleus during evolution. No known function can be assigned to the mung bean orf140, and we do not know whether it is actually expressed. The 5'-flanking region of orf140 also exists in a number of chimeric open reading frames found upstream or downstream from co-transcribed genes in mitochondria of various plant CMS lines, e.g. orf224 and orf158 in rapeseed (Bonhomme et al., 1992).

Northern-blot hybridization and RT-PCR showed that these four genes were co-transcribed. Additional transcription initiations may also exist and need to be further investigated. The 3'-ends of most transcripts are confined to within 300 bp downstream from the atp1 coding region since no signal can be detected by probe 5 (Figure 5, lane 5). A classic double-stemloop structure, which has been suggested to be involved in transcription termination or RNA processing in expression of plant mitochondrial genes, was not identified in our sequences (Schuster et al., 1986). 3'-termini not associated with stem-loop structures have also been reported in the past (Fauron and Casper, 1994).

Northern blotting also suggested that the largest 6.2 kb transcript is likely to be the precursor for the smaller transcripts detected. Among transcripts, the abundance of the 2.0 kb and 1.8 kb transcripts detected by probe 4, which is specific to atp1, is apparently higher than that of the 2.3 kb transcript detected by probes 2 and 3, which are specific to nad3 and rps12, respectively. If we assume that all the 2.3 kb, 2.0 kb and 1.8 kb transcripts are the processed products of the 5.0 kb transcript, different processing pathways or transcript stability have to be responsible for the levels of accumulation of these RNAs observed. Further investigation is required to elucidate the mechanism regulating expression of genes in this cluster, which encode products involved in different metabolism pathways in mung bean mitochondria. However, the same situation can also result from the use of alternative transcription initiation sites. Multiple transcription initiation sites have been observed in the protein coding genes coxIII and atp9 in maize (Mulligan et al., 1988) and in the rps3-rpl16-nad3-rps12 gene cluster of rice (Nakazono et al., 1995). In the case of rice, one transcription initiation site is used for transcribing the whole region of rps3-rpl16-nad3-rps12, but a second initiation site was also discovered between rps116 and nad3. Cap labeling assay and 5'- and 3'-end mapping of different transcripts detected here are required to further address this problem. Five independent cDNA clones of atp1 were isolated from a mitochondrial cDNA bank and were completely sequenced. Four editing sites were found among those cDNA clones (manuscript in preparation).

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綠豆粒線體基因群 orf140-nad3-rps12-atp1 之序列分析 及其轉錄核酸之分析

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由綠豆粒線體基因組中，克隆出 7.1 kb 之 DNA 片段，它包括有 orf140-nad3-rps12-atp1 之基因群。nad3, rps12 及 atp1 和其他植物序列有極高之相似性，orf140 及其 5' 端之核酸序列和向日葵及 *Oenothera* 之 ORFBs 序列相同。此基因群在粒線體基因組中屬單一基因。由北方墨點雜交及 PCR 所分析之結果，知此四基因為 co-transcribed 並可進一步分離為單一 transcripts。

關鍵詞：DNA 核酸序列；粒線體基因群；綠豆；orf140-nad3-rps12-atp1；基因轉錄。